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1) oredipe et al. proc soc exp biol med 194 / 4 : 301- 307 (1990)

2) szala et al. PNAS 87 / 17 : 6833 - 6837 (1990)

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Molecular cloning of cDNA for the human tumor-associated antigen CO-029 and identification of related transmembrane antigens

(COS cell expression/ME491/Sm23/CD37)

STANISLAW SZALA, YASUSHI KASAI, ZENON STEPLEWSKI, ULRICH RODECK, HILARY KOPROWSKI,
AND ALBAN J. LINNENBACH*

The Wistar Institute of Anatomy and Biology, 3601 Spruce Street, Philadelphia, PA 19104

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ABSTRACT The human tumor-associated antigen CO-029 is a monoclonal antibody-defined cell surface glycoprotein of 27-34 kDa. By using the high-efficiency COS cell expression system, a full-length cDNA clone for CO-029 was isolated. When transiently expressed in COS cells, the cDNA clone directed the synthesis of an antigen reactive to monoclonal antibody CO-029 in mixed hemadsorption and immunoblot assays. Sequence analysis revealed that CO-029 belongs to a family of cell surface antigens that includes the melanoma-associated antigen ME491, the leukocyte cell surface antigen CD37, and the Sm23 antigen of the parasitic helminth *Schistosoma mansoni*. CO-029 and ME491 antigen expression and the effect of their corresponding monoclonal antibodies on cell growth were compared in human tumor cell lines of various histologic origins.

A number of monoclonal antibodies (mAbs) have been derived from the immunization of mice with human gastrointestinal tumor cell lines (1-5). Investigations into the antigenic structures recognized by these mAbs have identified a group of glycolipid and glycoprotein antigens (2). The 40-kDa cell surface glycoprotein (6) recognized by mAb CO17-1A (7), and several other independently derived mAbs (4, 5, 8), is one of the most well-characterized tumor-associated antigens. Another cell surface glycoprotein antigen, defined by mAb CO-029 (formerly 1116NS-29) (1), has been shown to be a ~32-kDa monomer (9). The CO-029 antigen was found to be expressed on gastric, colon, rectal, and pancreatic carcinomas but not on most normal tissues (9). mAb CO-029 has been shown to mediate antibody-dependent cell-mediated cytotoxicity *in vitro* (1).

The recent molecular cloning of cDNA for tumor-associated antigens has provided information about antigen structure and the evolution of the genes encoding these antigens. cDNA clones have been isolated (10) for the GA733-2 carcinoma-associated antigen defined by mAb GA733 (7). Transfection experiments with a GA733-2 cDNA clone have shown that it encodes both the GA733 and CO17 1A epitopes (10). Sequence analysis of GA733-2 found it to be identical with the mAb-defined tumor-associated antigen KSA (11). A related gene, GA733-1, has been identified and was found to have a 50% amino acid sequence homology with the GA733-2 antigen (10, 12). GA733-1 is an intronless gene that was shown to be transcribed at high levels in pancreatic carcinoma cell lines (12). The GA733 family of antigens are type I transmembrane proteins of unknown function.

This report describes the immunoselection (13) of cDNA clones for the CO-029 antigen from an expression library constructed (10) from a human colon rectal carcinoma cell line.

CO-029 cDNA[†] was sequenced, and the amino acid sequence of the CO-029 antigen was predicted. Comparison of the CO-029 sequence with known sequences revealed that the CO-029 gene has a common ancestry with other genes encoding transmembrane antigens, including the human melanoma-associated antigen ME491 (14), the human leukocyte antigen CD37 (15), and the Sm23 antigen of the parasitic helminth *Schistosoma mansoni* (16).

MATERIALS AND METHODS

Immunoselection of cDNA Clones. A cDNA library (10) prepared from the SW948 colorectal carcinoma cell line was transfected into COS cells by the DEAE-dextran method (13). COS cells transiently expressing antigen were selected by "panning" (13) with mAb CO-029. Episomal DNA was recovered from the panned cells and transferred into *Escherichia coli* MC1061/P3 cells. Recombinant DNA was isolated from the *E. coli* cells and further enriched for CO-029 sequences by two additional cycles of immunoselection, with COS cell transfections being performed by spheroplast fusion (13) instead of DEAE-dextran. The first immunoselection of the total cDNA library yielded 11,600 drug-resistant bacterial colonies; 600 and 2900 colonies were observed after the second and third selections, respectively.

Analysis of Immunoselected Clones. Ten drug-resistant bacterial colonies resulting from the final selection were chosen at random and analyzed for cDNA insert size by digestion at flanking *Xho* I sites. DNA was prepared from a 1.5-ml bacterial culture of the enriched clone CO-029-5. One-fifth of this DNA was introduced into COS cells by the DEAE-dextran method. After a 3-day transient expression period, the transfectants were analyzed with mAb for the expression of the CO-029 epitope.

Transfected cells were assayed by the mixed hemadsorption assay (MHA) as described by Herlyn *et al.* (17). For Western blot analysis, cells were lysed in a buffer containing Nonidet P-40 (8), and 40 µg of nonreduced, nonheated, total protein was electrophoresed on a 12% polyacrylamide gel containing SDS (18). Proteins were electroblotted onto a nitrocellulose filter, which was incubated with affinity-purified CO-029 mAb at 1 µg/ml and then with affinity-purified goat anti-mouse IgG alkaline phosphatase conjugate and substrates for color development (Promega Biotec).

DNA Sequence Determination and Analysis. Both strands of the full-length cDNA clone CO-029-5 were sequenced by the dideoxynucleotide method (19). Sequence reactions were performed with T7 DNA polymerase (Pharmacia) using vector-specific and CO-029-specific primers. Compressions

Abbreviations: mAb, monoclonal antibody; MHA, mixed hemadsorption assay.

*To whom reprint requests should be addressed.

[†]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35252).

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were resolved by substituting 7-deaza-deoxyguanosine 5'-triphosphate for dGTP. The CO-029 sequence was tested for homology by searching release 63 of GenBank with the program TFASTA (20) and release 23 of the National Biomedical Research Foundation protein data base with the programs FASTP (21) and FASTA (20). Sequences with optimized scores >100 were further studied with the programs ALIGN (22) and LINE-UP (23).

CO-029 Expression in Tumor Cell Lines. Transcription of the CO-029 gene in human tumor cell lines was analyzed on RNA blots. Poly(A)⁺ cytoplasmic mRNA (2 μ g) (24) was electrophoresed on a 2.2 M formaldehyde/1% agarose gel (25) and transferred to nitrocellulose. The CO-029-5 cDNA clone was nick translated and hybridized to the filter in 0.05 M sodium phosphate, pH 6.5/1 \times Denhardt's solution (50 \times Denhardt's solution is 1% Ficoll 400/1% polyvinylpyrrolidone/1% bovine serum albumin)/0.1% SDS/50% (vol/vol) deionized formamide/5 \times SSC (20 \times SSC is 3.0 M sodium chloride/0.3 M sodium citrate, pH 7.0) at 42°C overnight. The filter was washed at high stringency in 0.1 \times SSC/0.1% SDS at 65°C. The probe was then removed and the filter was hybridized to a control probe, α -enolase cDNA (26).

Antigen expression in human tumor cell lines was determined by RIA (2). Equal numbers of cells were incubated with mAb CO-029 or ME491 (10 μ g/ml) and the binding was detected by ¹²⁵I-labeled rabbit anti-mouse IgG (Fab')₂ antibody.

Cell Growth Assays. [³H]Thymidine incorporation was measured after incubation of cells with purified mAbs in chemically defined serum-free medium. The colorectal carcinoma cell line SW948 and the melanoma cell line WM852 were cultured (100 μ l) in 96-well plates at 1.5×10^4 cells per well. mAbs CO-029 and ME491 (100 μ l per well at 5, 25, and 250 μ g/ml) were added 18 hr after seeding. After 24 hr, each culture was pulsed-labeled for 18 hr with 1 μ Ci of [methyl-³H]thymidine (48 Ci/mmol; 1 Ci = 37 GBq). Cells were treated with trypsin and collected with an automatic cell harvester (Skatron, Sterling, VA), and cell-associated radioactivity was determined in triplicate by liquid scintillation counting.

RESULTS

Expression of the Immunoselected cDNA Clone in COS Cells. Analysis of third-cycle recombinant bacteria indicated that 6 of 10 clones contained cDNA inserts 1.1 kilobase in size. Clone CO-029-5, representative of the enriched species, was introduced into COS cells by the DEAE-dextran method and expressed for 3 days. In MHA using mAb CO-029, $\approx 30\%$ of transfected COS cells formed rosettes (Fig. 1B), whereas untreated COS cells did not react (Fig. 1A). To confirm this result, Western blot analysis with the mAb was performed on COS cells transiently expressing the CO-029-5 clone. Transfected cells expressed a 27- to 34-kDa antigen (Fig. 2, lane 3), characteristic of the native CO-029 antigen expressed in control SW948 cells (Fig. 2, lane 1). Both cell types expressed several discrete forms of CO-029 between 27 and 34 kDa. Antigen was not detected in untreated COS cells (Fig. 2, lane 2). Thus, the CO-029-5 clone encoded the epitope recognized by the CO-029 mAb.

Sequence of CO-029 cDNA. The 1.1-kilobase sequence of CO-029-5 revealed an open reading frame for 237 amino acids beginning at the 5'-proximal ATG codon, which was found to be flanked by sequences similar to the consensus sequence for initiation of translation (27) (Fig. 3A). A protein molecular weight of 26,044 is predicted for CO-029. Thus, up to 8 kDa of the 27- to 34-kDa CO-029 glycoprotein may be accounted for by glycosylation (9). A single potential N-linked glycosylation site was observed (Fig. 3A). The 5' untranslated region consists of 138 residues; the 232-base 3' untranslated



FIG. 1. Detection of the CO-029 antigen in transfected COS by MHA. Cells were incubated with mAb CO-029 and then incubated with an indicator system consisting of sheep erythrocytes treated with mouse antiserum against sheep erythrocytes and goat anti-mouse IgG. (A) Untreated COS cells. (B) COS cells 3 days after transfection with CO-029-5 DNA.

region contained one consensus sequence for poly(A) addition and two sequences for mRNA turnover (28) (Fig. 3A).

Analysis of the distribution of hydrophobic and hydrophilic amino acids (29) (Fig. 3B) suggested that CO-029 is a type III integral transmembrane protein (30). Four hydrophobic stretches of ≈ 25 amino acids were found to be separated by hydrophilic amino acids (Fig. 3).

Homology of CO-029. Data base analysis revealed that the CO-029 antigen was related to the human melanoma-associated antigen ME491 (14) and the human leukocyte antigen CD37 (15). Other investigators have found ME491 to be homologous to the Sm23 antigen of the parasitic helminth

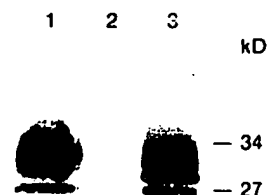


FIG. 2. Western blot detection of the CO-029 antigen in transfected COS cells. Total cell extracts were derived from control SW948 colorectal carcinoma cells (lane 1), untreated COS cells (lane 2), and COS cells 3 days after transfection with CO-029-5 DNA (lane 3).

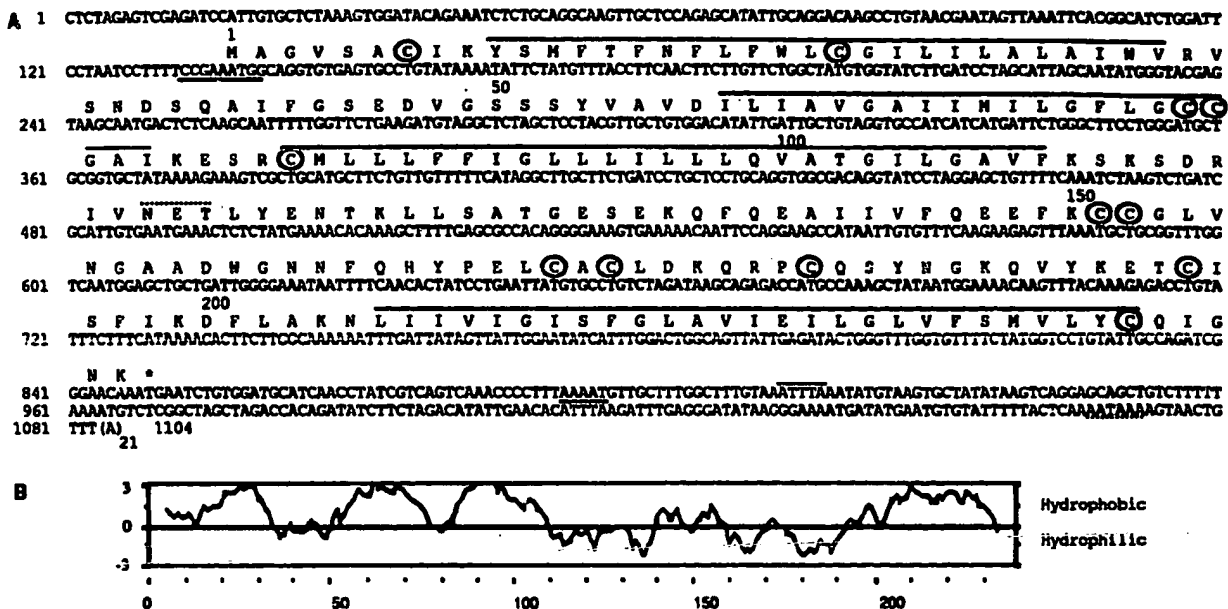


FIG. 3. (A) Sequence of full-length CO-029 cDNA with predicted amino acid sequence. The single-letter amino acid code is used. The positions of 12 cysteine residues (circles), a potential N-linked glycosylation site (dotted overline), and four hydrophobic regions (bold overline) are indicated. Consensus sequences for initiation of translation (underline), mRNA turnover (overline), and poly(A) addition (dotted underline) are shown. (B) Kyte-Doolittle hydrophobicity plot of the CO-029 antigen showing the four hydrophobic regions.

S. mansoni (16). Analysis of these sequences with the program ALIGN indicated that these homologies were statistically significant. For example, pairwise comparison of the CO-029 sequence with the ME491, Sm23, and CD37 sequences, by using a gap penalty of 40, resulted in alignment scores 34, 30, and 18 SD units above the mean score of 100 random runs, respectively.

A multiple sequence alignment indicated that the positions of cysteine and glycine residues were particularly well conserved in the CO-029 family (Fig. 4). Sequence homology was greatest in transmembrane and cytoplasmic domains predicted by hydrophobicity analysis (29) (data not shown). This homology was evident even in species as evolutionarily divergent as *Homo sapiens* and *S. mansoni*. For example, there were 10 consecutive amino acid identities occurring in CO-029 and Sm23 beginning at residue 74 of the alignment (Fig. 4). In contrast to the transmembrane and cytoplasmic domains, the extracellular domains probably have sustained

insertions and/or deletions. The sequences of the predicted extracellular domains were divergent, with the exception of the positions of cysteine residues. The number and positions of potential N-linked glycosylation sites varied among these antigens, but all positions corresponded to the major hydrophilic domains (Fig. 4).

Model for the CO-029 Family of Antigens. Two classes of antigen could be distinguished within this family. CO-029, ME491, and Sm23 proteins were found to be approximately equal in length and homologous throughout (Fig. 4). In contrast, the homology of CD37 to CO-029, ME491, and Sm23 was limited to the NH₂-terminal half of these antigens (Fig. 4).

If we assume that the CO-029, ME491, and Sm23 proteins have four transmembrane domains and that the positions of the potential N-linked glycosylation sites (Fig. 4) place the major hydrophilic domains outside the cell, then these antigens would be orientated so that the NH₂- and COOH-terminal ends would be on the cytoplasmic side of the

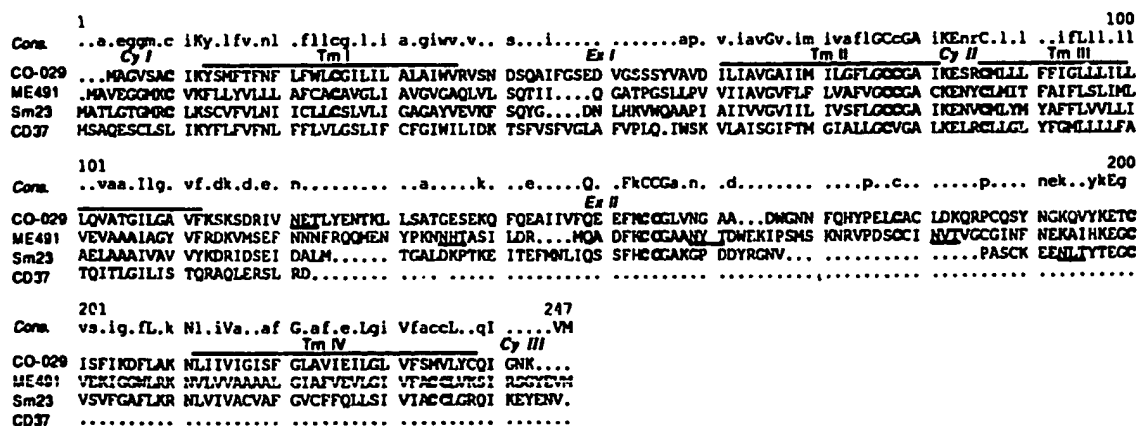


FIG. 4. Alignment of CO-029-related sequences. The CO-029, ME491, and Sm23 sequences are shown in their entirety; the homologous portion of the CD37 sequence is presented. The single-letter amino acid code is used. Conserved cysteine residues are in boldface type. Potential N-linked glycosylation sites (underline) and predicted transmembrane (Tm), extracellular (Ex), and cytoplasmic (Cy) domains are illustrated. Gaps (..) were inserted to maximize the alignment; a consensus sequence (Cons.) was calculated.

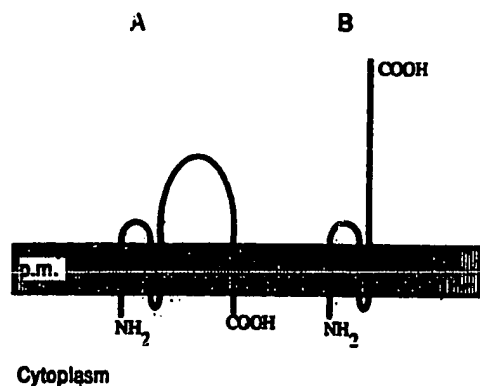


FIG. 5. Models for the CO-029 family of membrane antigens. Models: A, CO-029, ME491, and Sm23 antigens; B, CD37 antigen.

membrane (Fig. 5, model A). Two extracellular domains and three short cytoplasmic domains were predicted (Figs. 4 and 5, model A). The portion of CD37 homologous to the other CO-029-related antigens consisted of three transmembrane and two cytoplasmic domains (Figs. 4 and 5, model B).

Expression of the CO-029 Gene in Tumor Cell Lines. Transcription of the CO-029 gene was detected in the SW948 colorectal carcinoma and in the SW707 rectal carcinoma cell lines (Fig. 6A, lanes 1 and 2). The steady-state level of CO-029 mRNA differed in these two lines, whereas the level of α -enolase mRNA was approximately equal (Fig. 6B, lanes 1 and 2). The size of the full-length cDNA clone (Fig. 3A) correlated with the observed 1.15-kb CO-029 transcript. Two pancreatic carcinoma and two melanoma cell lines were negative for CO-029 mRNA (Fig. 6A, lanes 3–6). CO-029-related transcripts were not observed under the high-stringency hybridization and washing conditions used here.

CO-029 and ME491 antigen expression was compared in various tumor cell lines by RIA. Several cell lines coexpressed these genes (Fig. 7). The major qualitative difference in the expression of the two genes was that melanoma cell lines expressed the ME491 antigen but not the CO-029 antigen.

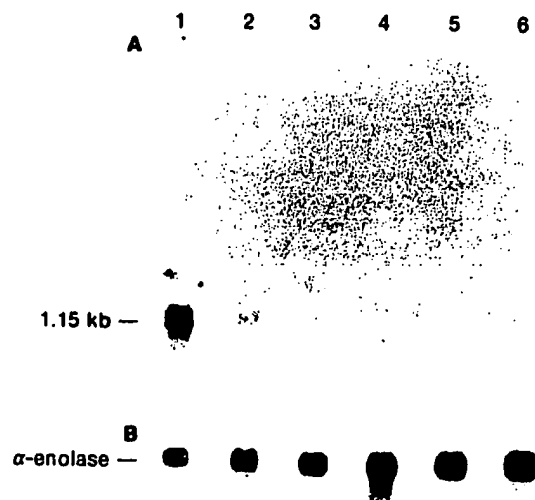


FIG. 6. (A) Northern blot analysis with the CO-029-5 DNA probe. mRNAs were derived from the SW948 colorectal carcinoma cell line (lane 1), the SW707 rectal carcinoma cell line (lane 2), the pancreatic carcinoma cell lines Capan-2 (lane 3) and BXP-3 (lane 4), and the melanoma cell lines WM1158 (lane 5) and WM35 (lane 6). (B) Hybridization with a control probe for α -enolase. kb, Kilobases.

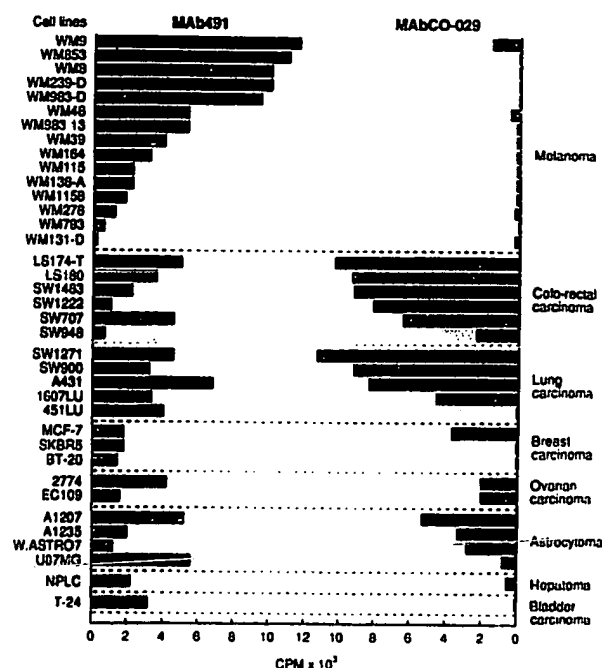


FIG. 7. Binding of mAbs ME491 and CO-029 in RIA to a panel of human tumor cell lines. Results are presented after subtraction of nonspecific mAb P3 binding (typically, 100–200 cpm).

In [³H]thymidine incorporation experiments to determine the effect of mAbs CO-029 and ME491 on the growth of human colorectal carcinoma and melanoma cell lines, no modulatory effects were observed (data not shown).

DISCUSSION

cDNA clones for the CO-029 tumor-associated antigen were isolated by immunoselection with mAb. Two experiments confirmed the identity of the CO-029 cDNA clone. Upon transfection of COS cells, which did not express the CO-029 antigen, a cDNA clone was shown by MHA to direct the synthesis of an antigen bearing the CO-029 epitope. Recombinant antigen expressed in transfected COS cells and native antigen expressed in a colorectal carcinoma cell line were indistinguishable by Western blot analysis.

Analysis of the predicted CO-029 amino acid sequence indicated that, to our knowledge, it was a heretofore unknown sequence with four potential transmembrane domains. CO-029 was found to be unrelated in sequence and membrane topology to the GA733 family of carcinoma-associated transmembrane antigens (10, 12). The CO-029 and GA733-2 antigens were found to be coexpressed in colorectal carcinoma cell lines and differentially expressed in pancreatic carcinoma cell lines, as demonstrated here and elsewhere (3, 10) by Northern blot analysis and RIA.

Common ancestry between the CO-029 gene and the genes for the melanoma-associated antigen ME491 (14) and the leukocyte antigen CD37 (15) was demonstrated here. ME491 has been described by other investigators (16) to be homologous to the *S. mansoni* antigen Sm23. Thus, there are now four known members of this family of type III transmembrane proteins.

Two types of gene duplications were apparent among CO-029-related genes. The CO-029, ME491, and Sm23 genes appear to have originated from the entire duplication of a common ancestral gene. Based on the boundaries of amino acid sequence homology, the CD37 gene was found to contain a partial duplication of the common ancestral gene.

Different patterns of tissue expression of the three human CO-029-related genes have been observed. It was shown here that the ME491 and CO-029 genes were coexpressed in several types of tumor cell lines but were differentially expressed in several melanoma cell lines. The CD37 antigen has been shown to be strongly expressed on B lymphocytes and at lower levels on other hematopoietic cell types.

The expression of a CO-029 homologue in an organism as primitive as the parasitic worm *S. mansoni* indicates that these genes are likely to be performing essential functions. Overall, these antigens share $\approx 30\%$ amino acid sequence identity. The distribution of sequence identities was found to be nonrandom. The lack of homology in the extracellular domains of the CO-029 family of antigens provided a strong indication that their functions are diverse. However, the finding of homology in their transmembrane and cytoplasmic domains suggested that these molecules share a common effector function. The 10 consecutive amino acid identities occurring between CO-029 and Sm23 may point out a domain critical to effector function.

Investigations into the biological function of these antigens have suggested that some antigens may be related to cell proliferation. In an *in vitro* model system using transfected mouse cells, the steady-state level of transcription of the ME491 gene was elevated by subsequent transfection with an activated H-ras oncogene (31). When cells expressing the ME491 antigen at the cell surface were treated with mAb ME491, the antigen was internalized (32). This phenomenon is analogous to the interaction between a mAb and its corresponding growth factor receptor (32). The proliferation of cultured human B cells has been shown to be modulated by mAb CD37 (33). In contrast, it was shown here that mAb CO-029 and ME491 do not modulate the growth of cultured tumor cell lines.

The molecular cloning of cDNA for tumor-associated antigens and the subsequent production of recombinant antigens may provide additional approaches to the experimental active immunotherapy of human tumors. In the future, it will be possible to compare the therapeutic potency of defined recombinant antigen vaccines with vaccines consisting of autologous tumor cell preparations (34) or anti-idiotypic antibodies bearing the internal image of tumor-associated antigens (35).

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